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Lupus-prone NZBWF1/J mice, defective in cytokine signaling, are resistant to fumonisin hepatotoxicity despite accumulation of liver sphinganine

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Abstract

Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium verticillioides*, commonly present in corn and other cereals. Exposure to FB_1 causes organ-specific diseases in various species, e.g., equine leukoencephalomalacia and porcine pulmonary edema; in mice the response is hepatotoxicity. We earlier reported that ceramide synthase inhibition by FB_1, the initial biochemical effect of this mycotoxin, results in modulation of cytokine network in response to accumulated free sphingoid bases. In the current study we used NZB/NZW-F1 (NZBW) mice that have modified cytokine expression and develop lupus beginning at 5 months of age. The NZBW and C57BL/6J (CBL) mice (appropriate control) were given five daily subcutaneous injections of either saline or 2.25 mg FB_1/kg/day and euthanized 24 h after the last treatment. Peripheral leukocyte counts were higher after exposure to FB_1 in CBL but not in NZBW. FB_1 treatment caused increases of plasma alanine aminotransferase and aspartate aminotransferase activity in CBL mice indicating hepatotoxicity; no elevation of circulating liver enzymes was recorded in NZBW mice. Hepatotoxic responses were confirmed by microscopic evaluation of apoptotic cells. The FB_1-induced proliferation of cells observed in CBL strain was abolished in NZBW animals. The sphinganine accumulation in liver after FB_1 was equal in both strains of mice. The NZBW strain lacked the FB_1-induced increases in the expression of liver tumor necrosis factor α, interferon γ, receptor interacting protein (RIP), and tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL), observed in CBL. Results confirmed our hypothesis that initial altered sphingolipid metabolism caused by FB_1 leads to perturbation of liver cytokine network and ultimate cellular injury; the mice deficient in cytokine signaling are refractory to FB_1 hepatotoxicity.

Keywords: Fumonisin B_1; Lupus mice; Sphinganine; Cytokines; Hepatotoxicity

1. Introduction

Interest in fumonisins, mycotoxins produced by *Fusarium verticillioides*, has increased because of

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their widespread prevalence in corn and corn products (WHO, 2000; Bolger et al., 2001). Fumonisin B_1 (FB_1), a congener of sphinganine, is one of the most potent and abundant member of this class and causes a variety of species-specific toxicological effects in domestic and laboratory animals (WHO, 2000; Bolger et al., 2001). It is a known cause of field outbreaks of porcine pulmonary edema (PPE) and equine leukoencephalomalacia (WHO, 2000; Bolger et al., 2001; Marasas, 2001). This mycotoxin is hepatocarcinogenic in male BDIX rats and female $B6C3F_1$ mice and a renal carcinogen in male F344/N rats (IARC, 2002). In all species tested it is hepatotoxic and in many nephrotoxic (Sharma et al., 1997). It has been implicated as a contributing factor in human esophageal cancer (Sydenham et al., 1990) and in primary liver cancer (Ueno et al., 1997).

The structural similarity of fumonisins with sphinganine led to the discovery of the initial biochemical effect of this group of mycotoxins, i.e., inhibition of de novo sphingolipid biosynthesis (Wang et al., 1991). FB_1 inhibition of ceramide synthase (sphinganine and sphingosine-*N*-acetyl transferase) leads to accumulation of sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley et al., 1996). Although the early effects of FB_1 (increased sphingoid bases and sphingoid base 1-phosphates or decreased complex sphingolipids) have been correlated with toxicity both in vitro and in vivo, how disruption of sphingolipid metabolism leads to cellular death is not clear. The downstream toxic mechanisms involved with fumonisins may be complex and involve several molecular sites. Cells exposed to FB_1 in vitro and in vivo undergo a mixture of necrotic and apoptotic cell death (Tolleson et al., 1996). We have previously demonstrated increased apoptosis in liver and kidney of mice after a short-term treatment with FB_1 (Sharma et al., 1997). The hepatopathy and nephropathy were closely correlated with the disruption of sphingolipid metabolism (Tsunoda et al., 1998).

Cell signaling by small lipid molecules has become a subject of intense research interest recently as different lipid messengers have been reported to possess survival as well as lethal signals (Maceyka et al., 2002). Ceramide, an important sphingolipid, produced by *N*-acylation of sphinganine and subsequent desaturation, or by hydrolysis of sphingomyelin, is known to induce apoptosis in cells (Pettus et al., 2002). However, the involvement of ceramide in the initiation or inhibition of the FB_1-induced apoptotic process is unclear (Tsunoda et al., 1998). The toxic property of ceramide is likely shared by sphingosine or possibly even sphinganine (Le Stunff et al., 2002). Because sphinganine is an intermediate in the de novo sphingolipid biosynthetic pathway, it is present in healthy cells at very low levels unless enzymes downstream, such as ceramide synthase, are blocked. FB_1 inhibition of ceramide synthase causes sphinganine levels to increase from picomolar to nanomolar or micromolar within a few hours (Yoo et al., 1996). Depending on the cell type, sphinganine-dependent cell death or cell proliferation is common outcome in cultured cells (He et al., 2001; Kim et al., 2001; Riley et al., 1999; Schmelz et al., 1998; Schroeder et al., 1994; Tolleson et al., 1999; Yu et al., 2001). The involvement of sphinganine as a cause of increased cell death or proliferation is verified by the fact that inhibitors of the first enzyme in the de novo sphingolipid biosynthetic pathway reduce sphinganine levels and prevent or reduce FB-induced cell death and proliferative effects (He et al., 2001). While free sphinganine is clearly involved in the cellular effects of fumonisins, decreased ceramide biosynthesis, elevated levels of sphingoid base 1-phosphates; decreased levels of glucosyl ceramide and decreased lipid raft associated glycosylphosphotidylinositol anchored membrane proteins are all potential contributors to the cellular consequences of disrupted sphingolipid metabolism (He et al., 2001; Kim et al., 2001; Merrill et al., 2001; Riley et al., 2001).

Our laboratory reported that tumor necrosis factor α (TNFα) is an important intermediary in the toxic responses to FB_1 in mice. In our earlier studies (Dugyala et al., 1998), we showed that (i) peritoneal macrophages derived from mice treated with FB_1 produce higher amounts of TNFα when stimulated by the mitogen, lipopolysaccharide (LPS), compared with controls, (ii) murine macrophage cells (J744A.1) treated in vitro with FB_1 produce TNFα, and (iii) acute in vivo hematological effects of FB_1 in mice are partially reversed by anti-TNFα antibodies. These observations strongly implied that TNFα was a potential mediator of fumonisin toxicity. Later we confirmed that FB_1-treated mice have increased expression of inflammatory cytokines in liver and kidney (Bhandari and Sharma, 2002) and mice lacking either of the TNFα receptors (TNFR-1 or TNFR-2) have decreased hepatotoxic responses to FB_1 (Sharma et al., 2001, 2000b).

A decreased hepatotoxicity after FB_1 exposure was also reported in a mouse strain with deleted interferon γ (IFNγ) gene (Sharma et al., 2003b). Conversely, decreased fumonisin hepatotoxicity was found in mice carrying the human tumor necrosis factor α transgene, whereas, TNF knockout mice exhibited increased hepatotoxicity (Sharma et al., 2000a, 2002). The involvement of TNFα pathway in FB_1 cytotoxicity has been confirmed by another laboratory; in African green monkey kidney (CV1) cells, the baculovirus inhibitor of apoptosis (CyIAP) and caspase inhibitors (crmA and p35) inhibited FB_1-induced apoptosis (Jones et al., 2001). In many studies using transgenic strains of mice sphinganine accumulation in liver correlated with the severity of the hepatotoxicity (Riley et al., 2001).

The NZW/NZB-F1 mice are genetically prone to the development of lupus and have been characterized to be defective in the production of proinflammatory cytokines; particularly in response to antigenic stimulus and have impaired clearance of apoptotic cells (Jongeneel et al., 1990; Sato et al., 2000; Theofilopoulos and Lawson, 1999). We hypothesized that the compromised cytokine regulation in lupus-prone mice will alter the hepatotoxic response of FB_1 in this strain. In the current study we investigated the effect of FB_1 on the expression of hepatic cytokines and associated hepatotoxicity in male lupus-prone mice and compared it to simultaneously treated C57BL/6J mice. The treatment protocol employed here was similar to that used previously (Sharma et al., 2000a,b,c, 2001, 2002, 2003b), in which the hepatotoxicity of FB_1 in male mice at this dose regimen has been well established. The sphingoid base accumulation in liver was also evaluated. We hereby report the insensitivity of the lupus-prone mouse strain to FB_1 hepatotoxicity in a short-term (5 days) exposure protocol despite the similarity of FB_1-induced accumulation in hepatic free sphinganine.

2. Materials and methods

2.1. Animals and housing

Male mice of strain NZBWF1/J (Stock No. 100008) were procured from Jackson Laboratories (Bar Harbor, ME). This strain was produced as a hybrid by crossing female NZB/BlNJ and male NZW/LacJ mice. These animals spontaneously develop systemic lupus erythematosus (SLE), a chronic inflammatory autoimmune disease. Since both parental strains are predisposed to the development of lupus, the control strain used in these experiments was C57BL/6J (Stock No. 000664), that has been used previously in FB_1-induced hepatotoxicity (Sharma et al., 2003b) and is phenotypically similar to the mutated strain. These animals have also been used as control for NZBW strain in other studies (Sawai et al., 2003; Theofilopoulos and Lawson, 1999). The lupus-prone NZBWF1/J animals are henceforth referred as NZBW, whereas the control C57BL/6J strain is indicated as CBL. All animals were 5 weeks old upon arrival and were acclimated for 10 days before experimentation. Mice were group-housed in individually ventilated cages under targeted controlled environmental conditions of 23 °C and 50% relative humidity with a 12-h light/dark cycle. Animals were provided free access to fumonisin-free food and tap water. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Treatment and sampling

Fumonisin B_1 (purity >98%) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Groups of five animals each were treated with five daily subcutaneous injections (1 ml/100 g body weight) of FB_1 in phosphate buffered saline (PBS), the dose per mouse being 0 (saline-treated controls) or 2.25 mg FB_1/kg/day. The animals were observed for any obvious behavioral changes and body weights were recorded daily. The dose and duration of treatment was selected based on our previous studies in which it produced a consistent and appreciable hepatotoxic damage in mice (Sharma et al., 2000a,b,c, 2001, 2002). Twenty-four hours after the fifth and the final injection, the animals were killed with halothane. Selected organs (liver, kidney, spleen and thymus) were isolated and weighed. Trunk blood was collected for hematological evaluations and plasma separated for liver enzyme activities. Liver samples were fixed in neutral formalin. Additional liver samples were quickly frozen on dry ice and stored at −85 °C until analyzed.

2.3. Hematology and liver enzymes in plasma

Total white and red cells were counted in blood using an electronic counter (Coulter, Hialeah, FL). Activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicators of hepatic damage, were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

2.4. Microscopy and TUNEL assay for apoptosis

Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4–5 μm), and stained with hematoxylin and eosin (H&E). The tissues were examined under a microscope in a random order and without knowledge of animal or group. Liver sections (5 μm) were also subjected to terminal dUTP nick end labeling (TUNEL) with a peroxidase-based In Situ Cell Death Detection® kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma et al., 2003a,b). The stained apoptotic cells were counted under a light microscope and normalized to the unit area as described (Sharma et al., 1997).

2.5. Enumeration of proliferating cell nuclear antigen (PCNA) positive cells

Hepatocyte proliferation was determined by analysis of PCNA in formalin-fixed, paraffin-embedded liver tissues as described recently (Sharma et al., 2003a,b). The number of PCNA positive cells were counted under a microscope and normalized to the unit area as described (Sharma et al., 1997). A few other cell types beside hepatocytes such as endothelial cells were labeled with PCNA, but only PCNA positive hepatocytes were counted.

2.6. Analyses of free sphingolipid bases in liver

Liver samples were analyzed for sphingoid bases. The method for extraction of free sphingoid bases was a modification of the method of Sullards and Merrill (2001). Briefly, 20 mg of tissue was combined with methanol/chloroform (2:1), sonicated and incubated at 48 °C. After 16 h, the mixture was cooled and 1 M methanolic KOH was added, sonicated, and incubated for 2 h at 37 °C. After 2 h the mixture was centrifuged and supernatant evaporated to dryness in a SpeedVac (ThermoSavant, Holbrook, NY). The residue was reconstituted in acetonitrile:water:formic acid (49.5:49.5:1) containing 5 mM ammonium formate and filter centrifuged. Sphingoid bases were chromatographically separated on a Thermal Separations (Riviera Beach, FL) high performance liquid chromatograph (HPLC) using an Intersil 5 μm ODS-3 column (150 mm × 3 mm, Metachem Technologies, Inc., Torrance, CA). The mobile phase was a mixture of 97% acetonitrile:2% water:1% formic acid (solvent A) and 97% water:2% acetonitrile:1% formic acid (solvent B) with a flow rate of 0.2 ml/min. The gradient started at 50% solvent A and at 15 min was 70% solvent A and at 20 min it was 100% solvent A which was held until 25 min at which time the column was re-equilibrated with 50% A. The column effluent was directly coupled to a ThermoFinnigan LCQ Duo ion trap mass spectrometer (MS, Thermo-Electron, Woburn, MA), operated in the electrospray ionization (ESI) positive ion mode with an inlet capillary temperature of 170 °C and nitrogen as sheath gas (50 arb). For MS/MS the collision energy was 30% and the parent *m/z* for MSMS were 300.2 and 302.2 for sphingosine, and sphinganine, respectively. For MSMS mass fragments were scanned from 195 to 400 *m/z* and compared to authentic standards purchased from Sigma Chemical (St. Louis, MO). Quantification was accomplished using internal standardization based on the recovery of C_{20}-sphinganine (*m/z* 330.2) added during extraction.

2.7. RNA isolation and ribonuclease protection assay (RPA)

Total RNA was isolated from frozen liver samples (ca. 70–80 mg) with TRI® reagent (Molecular Research Center, Cincinnati, OH), using a protocol described earlier (Bhandari and Sharma, 2002). Briefly, frozen tissues were ground in liquid nitrogen, TRI® reagent added and tissues further homogenized. The RPA was performed on samples using a RiboQuant™ RPA kit (Pharmingen, San Diego, CA). The template set included probes for cytokines e.g., tumor necrosis factor α (TNFα), TNFα-related apoptosis-inducing ligand (TRAIL), receptor interacting protein (RIP), lymphotoxin β (LTβ), interferon (IFN)γ, transforming growth factor (TGF) β1, and ribosomal protein L32 and glyceraldehyde phosphate dehydrogenase (GAPDH) as internal controls. The synthesis

of high specific-activity [α^{32}P]UTP-labeled T7 RNA polymerase-directed anti-sense RNA probes was done using the in vitro transcription kit according to the manufacturer's protocols (Pharmingen). An aliquot of 50 μg of sample RNA was hybridized with the probe overnight and digested with RNase A and RNase T1. The RNase-protected probes were purified and resolved on a denaturing polyacrylamide gel. The gels were exposed to a FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA) for 6–16 h and scanned by Bio-Rad Molecular Imager® FX. The relative gene expression was digitized using Quantity One® software provided by the manufacturer (Bio-Rad) and normalized against the house keeping gene, *L*32. The data were identical when normalized against GAPDH.

2.8. Statistics

Data from these studies were analyzed by a single-factor analysis of variance (ANOVA) followed by a post-hoc Duncan's multiple-range test. In the case of hepatic apoptosis and proliferating cell counts where unequal variances of different groups were observed the Wilcoxon Rank Sum test was employed. All tests were performed using a SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant; lower p values are indicated with respective results.

3. Results

3.1. The lupus mice were insensitive to hepatotoxic effects of FB_1

Treatment of both strains of mice with FB_1 had no effect on body weight changes or any other gross abnormality in behavior (data not presented). The erythrocyte counts were not influenced by the treatment; however, the total leukocyte counts were increased after FB_1 exposure in CBL mice only (Table 1). There was no treatment-related effect on liver weights; kidney weights were slightly reduced in NZBW animals. Spleen and thymus weights were not affected (not shown).

Treatment of CBL mice with FB_1 produced significant increases in activities of circulating liver enzymes (Fig. 1). Both ALT and AST showed an increase, indicative of FB_1-induced hepatic damage. In contrast liver enzymes were not significantly altered in the FB_1-treated NZBW mice compared to the saline-treated controls.

3.2. Changes in circulating liver enzymes were corroborated by microscopic and histochemical analyses

We compared the increases of circulating liver enzyme activities with liver pathology. Microscopic evaluation of H&E stained liver sections provided evidence of scattered apoptotic cells in liver of FB_1-treated CBL mice only (Fig. 2(a)). The architecture of these treated livers was otherwise indistinguishable form the livers of control mice. No leukocytic infiltration or hyperemia was evident. Occasional esinophilic staining of cytoplasm was observed. The apoptotic cells were single and not in clusters; sometimes groups of two or three cells were seen. These cells had fragmented or hyperchromatic or condensed nuclei occasionally separated from the cytoplasm. Increase of liver enzymes in CBL after FB_1 treatment suggests damage to hepatocytes although it was not apparent in microscopic examination. No FB_1-related changes

Table 1
Blood cell counts and relative organ weights in CBL and NZBW mice after fumonisin B_1 treatment

Strain, treatment	Blood cell counts		Relative organ weights, g/100 g body weight for	
	RBC (10^{-6}/mm^3)	WBC (10^{-3}/mm^3)	Liver	Kidney
CBL, saline	7.72 ± 0.32	3.64 ± 0.14	4.93 ± 0.13	1.44 ± 0.04
CBL, fumonisin	7.56 ± 0.48	5.46 ± 0.76*	4.88 ± 0.08	1.38 ± 0.04
NZBW, saline	6.80 ± 0.33	4.89 ± 0.21	4.43 ± 0.08	1.69 ± 0.03
NZBW, fumonisin	6.32 ± 0.27	4.88 ± 0.24	4.49 ± 0.16	1.47 ± 0.04**

Mean ± S.E., $n = 5$.
* $p < 0.05$ vs. saline-treated of the same strain.
** $p < 0.01$ vs. saline-treated of the same strain.

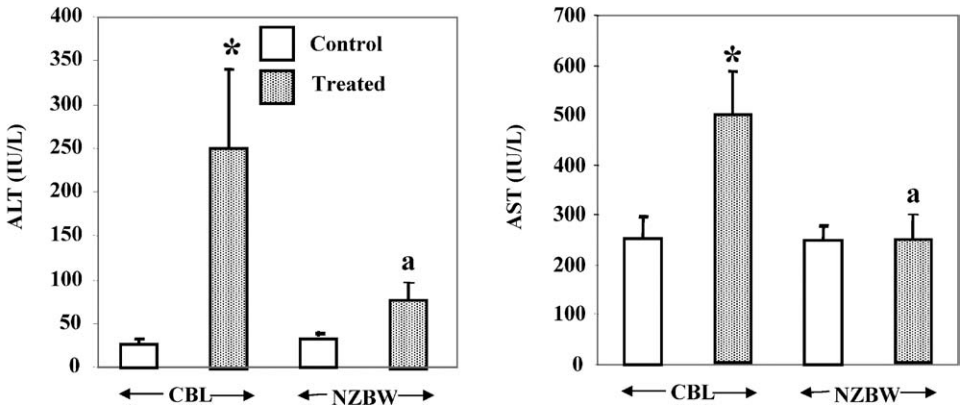

Fig. 1. Concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma of normal (CBL), and lupus-prone (NZBW) mice after treatment with FB_1 (mean ± S.E.M., $n = 5$). Significant difference between FB_1 and saline treatment of the same strain is indicated at $^{*}p < 0.05$. The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the FB_1-treated CBL and NZBW groups.

were observed in either saline treated groups or in FB_1-treated NZBW mice.

The liver sections were stained using TUNEL to confirm the apoptotic changes (Fig. 2(b)). The results of TUNEL evaluation are presented in Table 2. No TUNEL-positive cells were seen in any group other than the FB_1-treated CBL mice.

3.3. Lack of FB_1-induced hepatotoxicity in NZBW mice was not due to increased regeneration

The lack of FB_1 effect on livers of NZBW mice could be accounted for either by a lack of damage or a rapid regeneration of liver tissue. In order to check the regeneration process liver sections were stained with PCNA (Fig. 2(c)). Treatment of the CBL strain with FB_1 produced nearly a five-fold increase in PCNA-positive cells compared to the saline treated controls (Fig. 2(d)). The increase of PCNA-positive cells in FB_1-treated NZBW mice was not significant and was significantly lower than the number of PCNA positive cells in the FB_1-treated CBL mice, indicating that regeneration of liver in FB1-treated NZBW mice could not account for the lack of hepatotoxicity in this strain.

3.4. Sphinganine accumulation in liver of both mice strains in response to FB_1 was comparable

Results depicted in Fig. 3 indicate that FB_1 treatment increased the concentrations of free sphinganine to the same extent on sampling day in both CBL and NZBW strains. Neither of the strains had any increase in sphingosine content of liver after this relatively short duration of the treatment.

3.5. The FB_1 induction of inflammatory cytokines was not observed in lupus-prone mice

As the NZBW animals are reported to be deficient in cytokine production we measured production of various cell signaling molecules by RPA. This quantitative technique provides a measure of the expression of various genes and its validity has been established in our laboratory (Sharma et al., 2003a,b). The relative

Table 2
Incidence and number of apoptotic cells in mice treated with fumonisin B_1

Strain, treatment	Number of animals in group	Mice with apoptotic cells in liver	Number of cells/cm^2
CBL, saline	5	0	0
CBL, fumonisin	5	5	$41.9 \pm 16.5^{*}$
NZBW, saline	5	0	0
NZBW, fumonisin	5	0	0

* $p < 0.05$ vs. saline-treated of the same strain ($n = 5$).

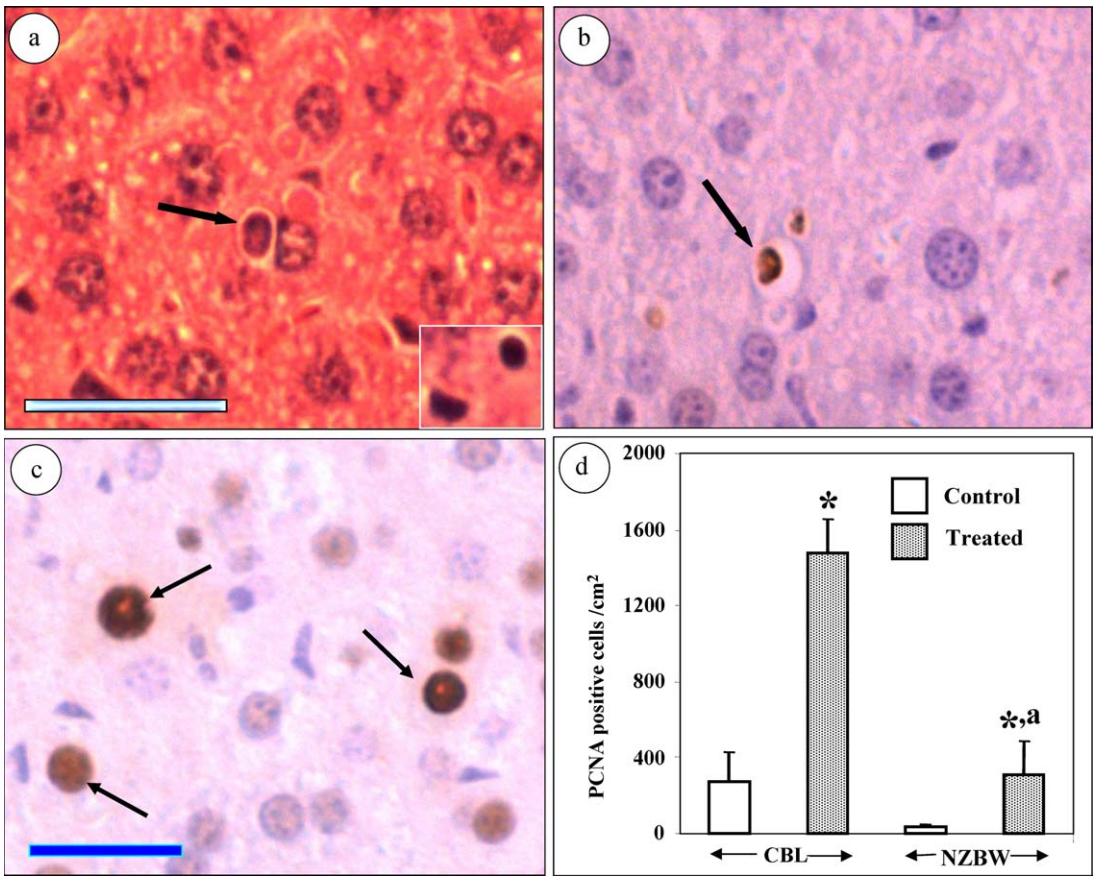

Fig. 2. (a) Photomicrographs (H&E) of liver showing FB_1-treated CBL mouse illustrating the hepatotoxic effects. Note the apoptotic cells (arrows), characterized by hyperchromatic nucleus of a hepatocyte separated from the cytoplasmic material. Inset in lower right illustrates a condensed and another crescent shaped nucleus; (b) liver form FB_1-treated CBL mice showing TUNEL positive cells. The TUNEL positive dark nucleus (arrow) is markedly different in texture and appearance from the normal ones, whereas TUNEL positive nuclear fragments (dark spots) are scattered in the tissue. Bar at the bottom left indicates 25 μm. Livers from NZBW showed no apoptotic cells after FB_1 treatment; (c) evaluation of cellular proliferation in liver showing the expression of PCNA by immunohistochemistry from a FB_1-treated CBL mouse showing normal architecture and several PCNA positive cells (marked by arrows). Bar in the lower right represents 25 μm; (d) the number of PCNA positive cells in tissue sections normalized to unit area (mean ± S.E., $n = 5$). * Indicates a significant difference from the saline-treated group of the same strain ($p < 0.05$). a, Significant difference ($p < 0.05$) form the FB_1-treated CBL.

expression of *TNFα*, *IFNγ* and TNFα-related signaling molecules, RIP and TRAIL, from FB_1-treated mice of the two strains is presented in Fig. 4. An increase in the expression of all of these apoptotic factors was seen in FB_1-treated CBL mice; however, no such increase was observed in NZBW mice after a similar treatment. The constitutive expression of these factors was however comparable in the two strains.

We also analyzed the expression of *TGFβ1* and *LTβ* in livers from animals in these experiments. Both of these molecules were increased in both strains to a similar extent (Fig. 5), although the increase of *TGFβ1* in the FB1-treated NZBW mice was not significantly different.

4. Discussion

Results obtained in these experiments indicate that NZBW mice are resistant to FB_1-induced hepatotoxi-

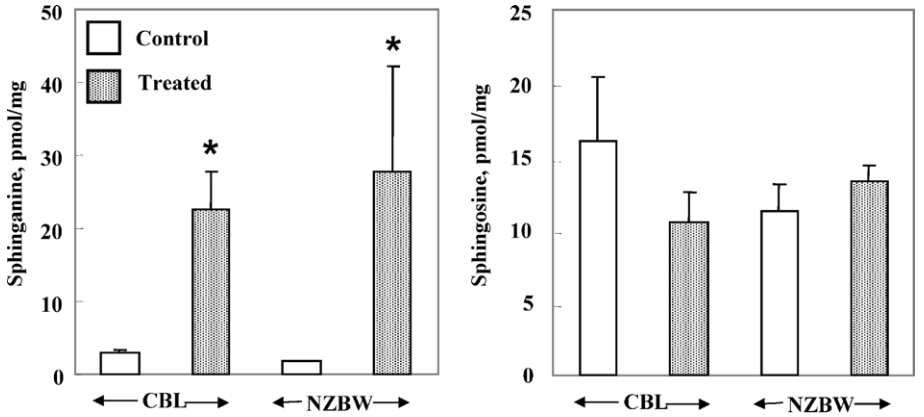

Fig. 3. Free sphinganine and sphingosine levels in the livers of CBL and NZBW mice after saline or FB_1 treatment (mean ± S.E.M., $n = 5$). Asterisks indicate a significant difference between FB_1 and saline treatment of the same strain at $^{*}p < 0.05$.

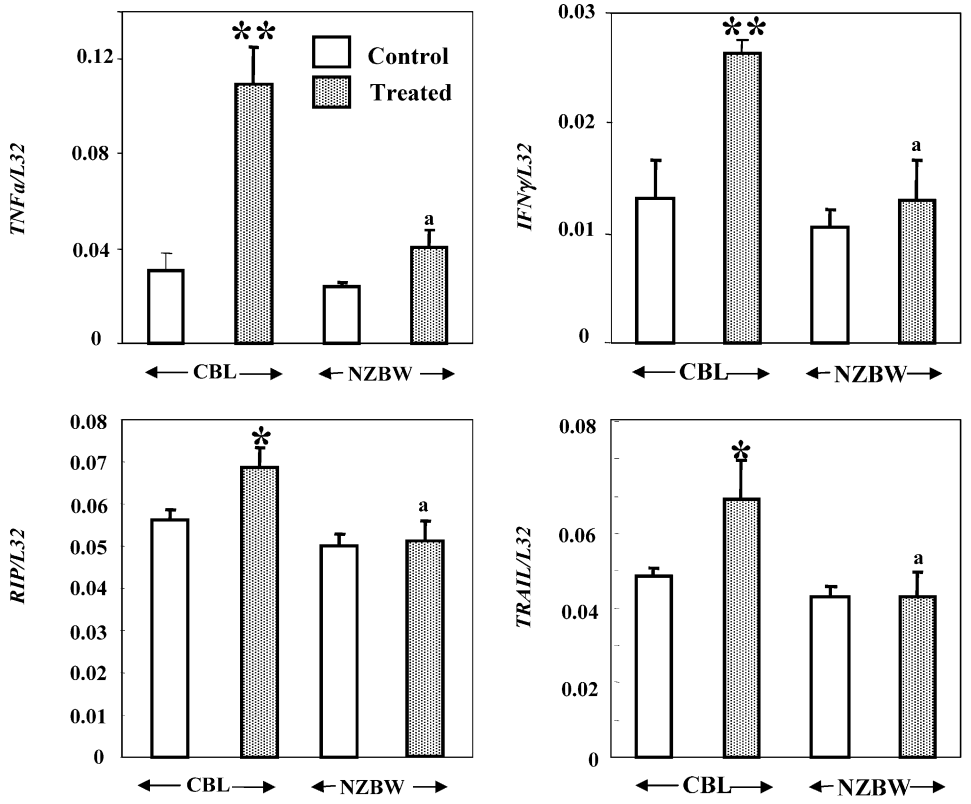

Fig. 4. The fumonisin B_1-induced changes in the mRNA expression for inflammatory cytokines *TNFα*, *IFNγ* and TNFα-related molecules *RIP* and *TRAIL*. The hepatic RNA form individual animal was subjected to RPA and normalized against *L*-32. mean ± S.E. ($n = 5$). Significant difference from the saline-treated group of the same strain at $^{*}p < 0.05$, $^{**}p < 0.01$. The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the FB_1-treated CBL and NZBW groups.

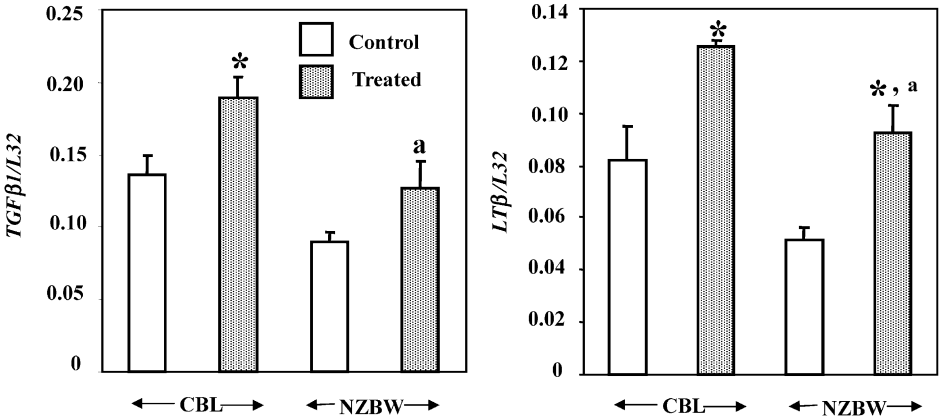

Fig. 5. The fumonisin B_1-induced changes in the mRNA expression for the growth factor *TGFβ1* and an apoptotic factor *LTβ*. The hepatic RNA form individual animal was subjected to RPA and normalized against *L*-32. Mean ± S.E. ($n = 5$). * Indicates a significant difference from the saline-treated group of the same strain at $p < 0.05$. The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the FB_1-treated CBL and NZBW groups.

city and increased cytokine expression than CBL mice despite the accumulation of comparable amounts of sphinganine in liver after 5 days of treatment with FB_1. The effects of FB_1 on liver of CBL mice were corroborated by activities of circulating liver enzymes, microscopic evaluation of liver and enumeration of TUNEL positive apoptotic cells in liver. The lack of overt hepatotoxicity in response to FB_1 in NZBW was not due to increased regeneration of liver in this strain since a reduction in the number of PCNA-positive cells was observed in livers of these animals. If liver damage was being actively repaired by regenerative proliferation, then increased PCNA labeling would have been observed.

The concentrations of free sphinganine in the liver of CBL and NZWB mice were similar and therefore the levels of free sphinganine in the liver after 5-day treatments are insufficient to explain the difference in hepatotoxicity in the CBL mice compared to the NZWB mice. Accumulation of free sphinganine is an indicator of ceramide synthase inhibition (Wang et al., 1991; Yoo et al., 1992); however, in cultured cells it has been shown that sphinganine only accumulates when the rate of sphinganine biosynthesis is greater than its rate of degradation or elimination (Enongene et al., 2002). The first step in sphinganine degradation is phosphorylation via sphingosine kinase to form sphinganine 1-phosphate. However, we did not observe increased accumulation of either sphinganine 1-phosphate or sphingosine 1-phosphate in CBL or NZWB mouse liver in this study (unpublished).

In the CBL mice there was elevation in inflammatory cytokines, as reported in previous studies (Bhandari et al., 2002; Sharma et al., 2003a,b). The FB_1-induced expression of inflammatory cytokines coincides with FB_1-induced hepatotoxicity, providing additional evidence for an important role for these cytokines in modulating FB_1-induced hepatotoxicity in mice; however, whether the elevation of cytokines is a cause or an effect is unknown. The fact that evidence of ceramide synthase inhibition (based on elevation in free sphinganine) is observed without hepatotoxicity or elevated cytokines could indicate that the NZWB mice are resistant to the toxic effects of disrupted sphingolipid metabolism or their threshold for hepatotoxicity is higher.

The role of inflammatory cytokines in FB_1 hepatotoxicity in mice has been suggested earlier from our laboratory. Bhandari et al. (2002) described the increase in TNFα and IFNγ in livers of BALB/c mice and speculated that a positive feed-back loop involving TNFα produced by Kupffer cells and IFNγ and IL-12 form T helper cells was involved, similar to that described for immunologic processes. The FB_1-induced hepatotoxicity was consistently reduced in mouse liver in models that lacked TNFα receptor (TNFR)1 or TNFR2 (Sharma et al., 2000b, 2001), and also IFNγ (Sharma et al., 2003b). Results obtained when mice strain lack-

ing TNFα was employed were unexpected; the hepatotoxicity and elevation in free sphinganine in this transgenic strain after treatment with FB_1 was exacerbated (Sharma et al., 2002). The latter effect was later described due to induction of *Fas* (*CD95*) in livers of the mutant strain, this response was not observed in normal intact mouse liver (Sharma et al., 2003a). Interestingly, activation-induced apoptosis of T-cells from humans predisposed to SLE are defective in Fas expression and normal levels of apoptosis can be restored by exposure to C-2 ceramide (Stassi et al., 1997). In another study (Sakata et al., 1998), immobilized Fas ligand-induced T-cell proliferation in SLE patients could also be induced by ceramide and the response was inhibited by FB_1; suggesting that the de novo ceramide pathway is involved in Fas-transduced signals in SLE T-cells.

The NZBW hybrid mice are used as a model for studying the pathogenesis of systemic lupus erythematosus (SLE), a chronic, inflammatory, autoimmune disease of unknown origin (Theofilopoulos and Dixon, 1985). NZBW mice develop an autoimmune disease resembling human SLE. Autoimmunity is characterized by high levels of antinuclear antibodies, hemolytic anemia, proteinuria, and progressive immune complex glomerulonephritis. The average lifespan for NZBW is 245 days for females and 406 days for males. These mice have an aberrant expression of TNFα (Jongeneel et al., 1990) and IL-12 (Liu and Beller, 2002). An IFN-inducible gene, *Ifi*202, has been linked to the onset of SLE (Rozzo et al., 2001). These animals are deficient in induced-apoptosis of immunocompetent cells or in the disposal of apoptotic cells (Potter et al., 2003). Our results also suggest that cells in these mice are incapable of undergoing apoptosis in response to FB_1, perhaps due to abnormal modulation of cytokines. The constitutive expression of TNFα and IFNγ were, however, comparable in the two strains used here. Nonetheless, the FB_1-induced increase was observed only in CBL mice. Whether the NZBW mice will develop hepatic tumors in response to FB_1 is not known.

Fumonisin B_1 increased the expression of TNFα-related signaling molecules in livers of CBL mice. Both RIP and TRAIL are increased in CBL only, no effect of FB_1 was observed in NZBW animals. Induction of these signaling agents by FB_1 was similar to that observed earlier (Bhandari and Sharma, 2002). The reduced proliferation in FB_1-treated livers of NZBW mice as observed by reduced PCNA-positive cells compared to that in CBL was in agreement with a lack of induction of *TGFβ*1 in the lupus-prone mice. The effect of FB_1 on *LTβ* may be of less consequence as this cytokine was increased after FB_1 in both mouse strains to a comparable extent.

The NZBW mice are in general not incapable of producing inflammatory cytokines. The secretion of IFNγ from concanavalin A (Con A)-stimulated spleen cells of mice infected with *Plasmodium chabaudi* was greater in NZBW animals than from cells of BALB/c mice (Sato et al., 2000). Even the Con A-stimulated production of IFNγ was higher in cells from the non-infected lupus-prone animals in this report than those from BALB/c; the effect was somewhat opposite on the secretion of IL-2. This suggests that somehow the modulation of inflammatory cytokines in the lupus-prone animals in response to exogenous stimuli, including FB_1, is deregulated; perhaps via a defect in the linkage between signaling pathways involving de novo ceramide generation (Sakata et al., 1998; Stassi et al., 1997).

It has been shown that in vitro and in vivo effects of FB_1 involve oxidative damage resulting in activation of phospholipase A_2 (PLA_2) and depletion of reduced glutathione (Abel and Gelderblom, 1998; Stockmann-Juvala et al., 2004). There is ample evidence that TNFα-mediated pathways activate PLA_2 and increase lipid peroxidation in hepatocytes causing depletion of reduced glutathione (Adamson and Billings, 1993; Adamson et al., 1994). Because of lack of modulation of inflammatory cytokine pathways in NZBW strain it is likely that FB_1 may not induce oxidative damage and consequent hepatotoxicity in these animals.

The aberrant signaling of TNFα and TNFα receptor 1 was recently reported in a related lupus-prone strain, NZM2410 (Blenman et al., 2004). In this study the authors investigated an endotoxicosis model involving lipopolysaccharide and D-galactosamine. The NZM2410 animals were resistant to endotoxicosis characterized by circulating ALT, the appearance of apoptotic cells in liver, and mortality compared to B6 mice. The lupus-prone animals had a significantly less TNFα in serum after administration of LPS and also significantly less TNFα-induced production of IL-6 and IL-10.

Although the results of current and several former reports imply that inflammatory cytokines are medi-

ators of FB_1 hepatotoxicity in mice and this effect is correlated with accumulation of free sphinganine in cells, the mechanism as how disrupted sphingolipid metabolism increases the cytokine production is still not understood. In addition to the effect of FB_1 on cytokine production, this mycotoxin also modulates the activity of protein kinase C (PKC) and various mitogen-activated protein kinases (MAPKs) that are important in cell signaling and also cytokine induction. The activation of PKC in the short-term by FB_1 was reported in brain slices (Yeung et al., 1996); the effect was specific for PKCα isoform in LLC-PK_1 cells (Gopee and Sharma, 2004). On the other hand FB_1 later repressed the PKC activity (Huang et al., 1995); several isoforms of PKC were effectively inhibited in LLC-PK_1 cells (Gopee et al., 2003). Pinelli et al. (1999) reported the activation of MAPK cascade in a human epithelial cell line. In preliminary studies with LLC-PK_1 cells such effect was observed primarily on c-jun N-terminal kinase (Johnson et al., 2003). Since PKC and MAPK induce various cytokines by involving the activation of nuclear factor κB (NFκB) and lupus-prone mice have reduced activation of NFκB (Jolly et al., 2001), it may be speculated that the reduced induction of cytokines in the NZBW strain is related to their decreased NFκB activity.

In conclusion, results presented here demonstrate that lupus prone NZBW mice are resistant to FB_1 induced hepatotoxicity and FB_1-induced cytokine expression than the CBL and other mouse strains that have been studied using a similar treatment protocol. In addition, the NZBW appear to be less responsive to disrupted sphingolipid metabolism; suggesting that there may be a defect in the linkage between disruption of sphingolipid-dependent signaling pathways that lead to increased apoptosis and cytokine expression in FB_1-treated mice. One of these signaling pathways includes inflammatory cytokines that can induce each other in liver. More research is needed to determine the active molecule and how it can initiate the cytokine cascade.

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